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Identification by Flow Cytometry of Seiridin, One of the Main Phytotoxins Produced by Three *Seiridium* Species Pathogenic to Cypress

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ABSTRACT Seiridin (SE), one of the main phytotoxins produced in vitro by *Seiridium* species pathogenic to cypress, was oxidized and the corresponding ketone derivative covalently linked to bovine serum albumin (BSA). The conjugate (SE-BSA) was used to prepare an antiserum to SE. The antibodies were absorbed with BSA and their specificity was assayed by ELISA and flow cytometry against SE, *iso*-seiridin (ISE), a structural isomer of SE, and some derivatives of these two metabolites. The antibodies tested in a competitive indirect ELISA did not show any binding activity to SE, ISE and their derivatives. The cytometryc test, instead, was successful. SE-BSA and SE showed the highest binding activity with the antibodies. SE derivatives having a shift on the adjacent carbon, oxidation, or acetylation of the hydroxy group of the heptyl side chain at C-4 or conversion of the γ-lactone in the corresponding planar furane ring reacted less than SE. The 2′-dansylhydrazoneSE and the 3,4-dihydroSE having a bulky group attached to the heptyl side chain and a saturated lactone ring, respectively, showed a weak reactivity. SE derivatives in which the γ-lactone ring was destroyed and ISE derivatives presenting the shift of the hydroxy group at C-3′ and another structural modification had no binding activity. *Nat. Toxins 5:14–19, 1997.*

Key Words: *Seiridium;* cypress canker disease; seiridins; phytotoxins; antibodies; seiridin derivatives; cytometry

INTRODUCTION

Seiridin (SE, **1**) and its structural isomer *iso*-seiridin (ISE, **2**) are two $\Delta^{\alpha,\beta}$-butenolides 3,4-dialkylsubstituted isolated as the main phytotoxins from the culture filtrates of three species of *Seiridium* (*cardinale, cupressi,* and *unicorne*). These are fungi associated with a canker disease of cypress (*Cupressus sempervirens L.*) in the Mediterranean area [Sparapano et al., 1986; Evidente et al., 1986]. The two butenolides are produced in vitro together with three toxic sesquiterpenoids, named seiricardines A, B, and C, and the 7′-hydroxyseiridin and 7′-hydroxyisoseiridin, recently characterized as two new $\Delta^{\alpha,\beta}$-butenolides 3,4-dialkylsubstituted closely related to **1** and **2**; moreover, cyclopaldic acid and the 14-macrolide seiricuprolide are produced only by *S. cupressi* [Evidente and Sparapano, 1994]. The total enantioselective synthesis of seiridin has also been realized [Bonini et al., 1995].

A study on the structure-activity relationships of some derivatives of seiridin and *iso*-seiridin carried out assaying the phytotoxicity, the antimicrobial and the hormone-like activities, showed that the integrity of the $\Delta^{\alpha,\beta}$-unsaturated-γ-lactone ring and the location of the hydroxy group of the heptyl side chain of SE are important for the biological activity of the two butenolides [Sparapano and Evidente, 1995].

The role that seiridins play in the pathogenesis of the canker of cypress has not been established [Graniti and Sparapano, 1990; Sparapano et al., 1993a,b)]. First, it should be ascertained if seiridins accumulate in the infected tissues of the host-plant. For this purpose, analytical methods which allow the specific detection of the seiridins at low concentration may be useful. Chromatographic methods that have been used to detect seiridins in *Seiridium* culture filtrates [Sparapano et al., 1994] failed when the toxins were present at low levels as in the extracts of infected cypress tissues.

This paper describes the attempt to identify seiridin by using ELISA and flow cytometry. This latter technique has

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been used to measure DNA content in cells of animals, plants and fungi [de Vita et al., 1994; Galbraith et al., 1983], analyze the cell cycle [Belloc et al., 1994; Kallioniemi et al., 1994] and detect membrane and intracellular antigens [Sumner et al., 1991; Wing et al., 1990]. In plant virology flow cytometry has been used for the detection of cucumber mosaic virus [Iannelli et al., 1996].

To apply the method antibodies raised against SE conjugated with bovine serum albumin were used. The specificity of these antibodies was tested by comparing their binding with SE and 7 of its derivatives as well as with ISE and 6 of its derivatives in a competitive indirect assay.

MATERIAL AND METHODS

Chemical Methods

Analytical and preparative thin layer chromatographies (TLC) were performed on SiO_2 (Merck, Kieselgel 60 F_{254}, 0.25 and 0.50 mm, respectively) plates; the spots were visualized by exposure to UV radiation or by spraying with 10% H_2SO_4 in MeOH and then with 5% phosphomolybdic acid in MeOH followed by heating at 110°C for 10 min.

Instrumentation

Infrared (IR) were obtained on a Perkin-Elmer (Oak Brook, IL) FT 1720X spectrometer. Ultraviolet (UV) spectra were taken on a Perkin-Elmer Lambda 7 UV/Vis spectrophotometer in MeCN solutions. ^1H and ^{13}C nuclear magnetic resonance (NMR) spectra were recorded in $CDCl_3$ at 270 and 67.92 MHz, respectively, on a Bruker spectrometer (Karlsruhe, Germany), using the same solvent as internal standard. Electron ionization mass spectra (EIMS) were recorded on a Fisons TRIO-2000 (VG Organic, Manchester, U.K.).

Production of Seiridin, *Iso*-seiridin, and their Derivatives

The structures of seiridin (SE), *iso*-seiridin (ISE), and their derivatives, designated with numbers from **1** to **15**, are shown in Figure 1.

SE and ISE were obtained as pure oil by chromatographic purification from the organic extract of culture filtrates of *S. cupressi* as previously described [Ballio et al., 1991].

The 2′-*O*-acetylSE (**3**) and 3′-*O*-acetylISE (**4**); the 3,4—dihydroSE (**5**) and 3,4-dihydroISE (**6**) and SE- and ISE-$LiA1H_4$ reduction products (**7** and **9** and **11** and **12**); and their corresponding triacetylderivatives (**8** and **10**) were prepared from **1** and **2** as previously reported [Evidente et al., 1986]. The preparation and the chemical characterization of 2′-dansylhydrazoneSE (**15**) will be reported elsewhere.

2′-Oxoseiridin (13)

Seiridin (100 mg) dissolved in dry CH_2Cl_2 (68 ml) was oxidized with the Corey's reagent (675 mg) at room temperature under stirring as previously described [Corey and Suggs, 1975]. After 5 hr, as all seiridin was converted into a lesser polar product (R_f 0.34 and 0.59, respectively, by TLC, eluent $CHCl_3$-*i*-PrOH 95:5) the reaction was stopped by addition of dry Et_2O and filtration on a short SiO_2 column. The colourless solution was dried under reduced pressure and the residue (98 mg) purified by preparative TLC ($CHCl_3$-*i*-PrOH 95:5) to yield 2′-oxoseiridin (**13**) as a homogeneous oil (74 mg); UV λ_{max} nm (log ϵ): 213 (4.20); IR ν_{max}, cm^{-1}: 1,751 (C = O, lactone), 1,713 (C = O, ketone), 1,676 (C = C), 1,167, 1,080 (O-CO); ^1H-NMR, δ: 4.64 (2H, br q, $J_{5,8'}$ = 1.9 Hz, H-5), 2.44 (2H, br t, $J_{6'7'}$ = 7.1 Hz, H-7′), 2.41 (2H, t, $J_{3',4'}$ = 7.9 Hz, H-3′), 2.14 (3H, s, H-1′), 1.82 (3H, br t, $J_{5,8'}$ = 1.9 Hz, H-8′) 1.60 (2H, m, H-6′), 1.50 (2H, m, H-4′), 1.34 (2H, m, H-5′); ^{13}C-NMR, δ: 208.3 (C-2′, s), 175.2 (C-2, s), 160.1 (C-4, s), 122.7 (C-3, s), 71.2 (C-5, t), 43.1, (C-3′, t), 29.7 (C-1′, q), 28.8 (C-4′, t), 27.3 (C-6′, t), 26.8 (C-7′, t) 23.1 (C-5′, t), 8.3 (C-8′, q). EIMS, *m/z*, (relative intensity): 210 $[M]^+$ (31), 195 $[M\text{-}Me]^+$ (2), 182 $[M\text{-}CO]^+$ (3), 167 $[M\text{-}Me\text{-}CO]^+$ (15), 149 $[M\text{-}Me\text{-}CO_2]^+$ (13), 125 $[M\text{-}C_5H_9O]^+$ (67), 112 $[M\text{-}C_6H_{10}O]^+$ (100).

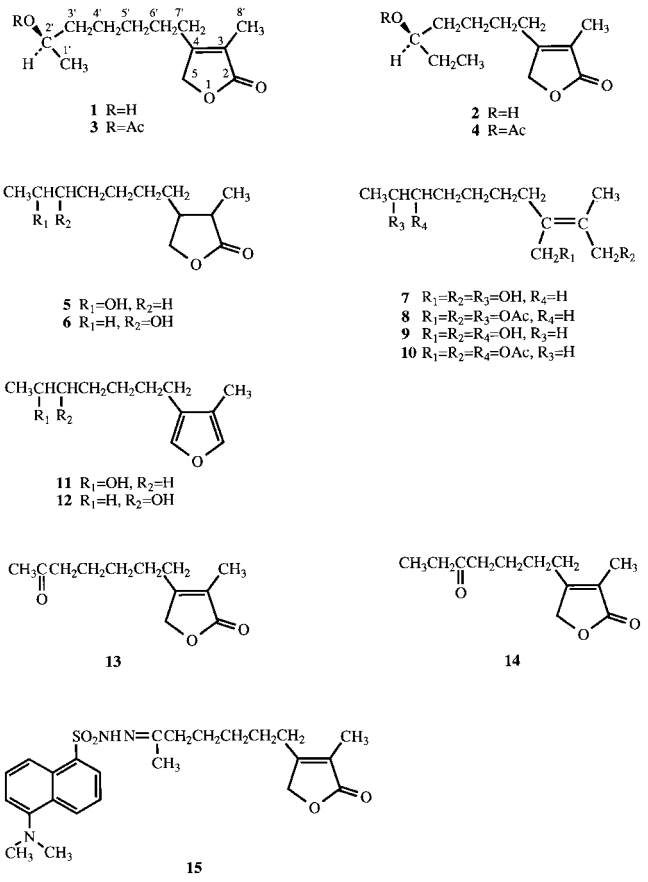

Fig. 1. The structure of SE (**1**), ISE (**2**), and of their derivatives (**3, 5, 7, 8, 11, 13, 15,** and **4, 6, 9, 10, 12, 14,** respectively).

3′-Oxo*iso*seiridin (14)

A sample of *iso*-seiridin (27 mg) was converted into the corresponding 3′-oxoderivative (**14**) as previously described

TABLE I. Detection of Seiridin by the Inhibition Cytofluorimetric Test

Anti-SE-BSA dilution	Relative change in sample fluorescence intensity[a]								
	SE-BSA			SE			ISE		
	0[b]	10[b]	50[b]	0[b]	10[b]	50[b]	0[b]	10[b]	50[b]
10^{-3}	480 ± 4.1	102 ± 0.9	97 ± 0.7	495 ± 4.2	330 ± 2.3	310 ± 2.1	501 ± 4.7	355 ± 3.4	332 ± 2.6
5×10^{-3}	320 ± 2.2	71 ± 0.8	75 ± 1.0	317 ± 1.4	196 ± 1.1	190 ± 1.4	317 ± 1.4	265 ± 2.1	262 ± 1.8
10^{-4}	92 ± 0.7	25 ± 0.4	19 ± 0.2	88 ± 0.9	68 ± 0.6	61 ± 0.7	85 ± 0.8	75 ± 0.8	72 ± 0.6

[a]Values (mean channel of fluorescence of the sample subtracted by the mean channel of the control) are the average of three experiments ± the standard deviation.

[b]Concentration (μg/ml) of SE-BSA, SE, and ISE used in the test.

to oxidize **1** to **13.** The crude product was purified by preparative TLC ($CHCl_3$-*i*-PrOH 95:5) to give the 3′-oxoisoseiridin (**14**) as homogeneous oil (17 mg); UV λ_{max} nm (log ϵ): 212 (4.42); IR ν_{max}, cm^{-1}: 1,748 (C = O, lactone), 1,713 (C = O, ketone), 1,677 (C = C), 1,116, 1,081 (O-CO); ^{1}H NMR, δ: 4.64 (2H, br q, $J_{5,8'}$ = 1.9 Hz, H-5), 2.43 (2H, br t, $J_{6',7'}$ = 6.8 Hz, H-7′), 2.41 (2H-2′, q, $J_{1',2'}$ = 6.9 Hz, H-2′), 2.40 (2H, t, $J_{4',5'}$ = 7.8 Hz, H-4′), 1.80 (3H, br t, $J_{5',8'}$ = 1.9 Hz, H-8′), 1.60–1.47 (4H, m H-5′ and H-6′), 1.04 (3H, t, $J_{1',2'}$ = 6.9 Hz, H-1′); EIMS, *m/z*, (relative intensity): 210 $[M]^+$ (20), 181 $[M\text{-}C_2H_5]^+$ (12), 153 $[M\text{-}C_2H_5\text{-}CO]^+$ (11), 138 $[M\text{-}C_2H_5\text{-}CO\text{-}Me]^+$ (95), 125 $[M\text{-}C_5H_9O]^+$ (95), 112 $[M\text{-}C_6H_{10}O]^+$ (100).

Preparation of the Immunogen

An aliquot of 2′-oxoseridin (22 mg) dissolved in dioxan (3 ml) was added to a solution of bovine serum albumin (BSA, 25 mg) (Sigma, St. Louis, MO), dissolved in 10^{-4} N HCl (6 ml). The mixture was left at 70°C under stirring for 3 days, neutralized with 0.04 N NaOH, and then treated with $NaBH_4$ (164 mg). Reduction was performed under stirring for 24 hr and stopped by dialysis in tubes with a molecular weight cut-off of 12,000–14,000 daltons for 2 days against a large volume of H_2O (1:10) with frequent changes. Finally, the tube contents were lyophilized yielding a white cotton product (23 mg).

Preparation of Antibodies

SE conjugate with BSA (SE-BSA) was used to immunize two New Zealand rabbits following the procedure previously described [Del Sorbo et al., 1994].

In order to purify antibodies specifically recognizing SE, the antiserum was mixed with BSA (10 to 100 μg/ml antiserum) and incubated overnight at 4°C. The mixture was centrifuged for 20 min at 10,000*g* and the pellet discarded.

Competitive Indirect Enzyme-Linked Immunosorbent Assay

The procedure followed was previously described [Del Sorbo et al., 1994]. Different dilutions (1:10 to 1:1,000) of absorbed antibodies were incubated with varying amounts (0.1 to 1,000 μg/ml) of SE, ISE or their derivatives.

Standard Cytofluorimetric Test

A total of about 10^7 latex particles (Polyscience, Eppelheim, Germany) were incubated overnight at 4°C under agitation with 1 ml of a SE-BSA solution (0.5, 5.0, or 50 μg/ml). The rest of the assay was carried out at room temperature. The size of the particle was 3 μm and the number of particles per tube 10^6. The mixture was centrifuged and the pellet incubated for 30 min with 1% gelatin in 0.2 M borate buffer, pH 8.5. The particles were then washed with 0.15 M phosphate buffered saline pH 7.2 (PBS) and incubated for 4 hr with anti-SE-BSA diluted in PBS, washed with PBS, and incubated with goat anti-rabbit immunoglobin labelled with fluorescein (anti-R^{FITC}) (Sigma Chemical Co., St. Louis, MO) for 1 hr. Particles were washed once with PBS and tested at the flow cytometer. Controls were incubated with PBS instead of anti-SE-BSA. The instrument (FACScan, Becton-Dickinson, San Jose, CA, USA) was equipped with a 15 mW, air cooled 488 nm argon ion laser. Green fluorescence (FITC) was collected through a 530/30 nm bandpass filter. The data of 10,000 events were collected for each sample, stored in list mode, and analyzed using Consort 32 system (Hewlett-Packard, Sunnyvale, CA). Forward (FSC) and side (SSC) scattering were analyzed on a linear scale; FITC fluorescence on a logarithmic scale. No gates were set around the particles. Results are presented as the mean channel fluorescence of the sample subtracted by the mean channel of the control. The autofluorescence (the average fluorescence intensity of control tubes) varied between 1.5 and 2%.

Competitive Indirect Cytofluorimetric Test

The capacity of antibodies to interact with the SE, ISE, and their derivatives was measured by a competitive indirect assay. For this purpose different dilution of toxins and derivatives (50 μl of 0.5, 5.0, 10, and 50 μg/ml) were incubated overnight with antibodies (50 μl diluted 1:1,000, 1:5,000 or 1:10,000). The pool was assayed by the standard cytofluorimetric assay.

RESULTS AND DISCUSSION

We propose a specific and sensitive method for the identification of seiridin, one of the main toxins produced by

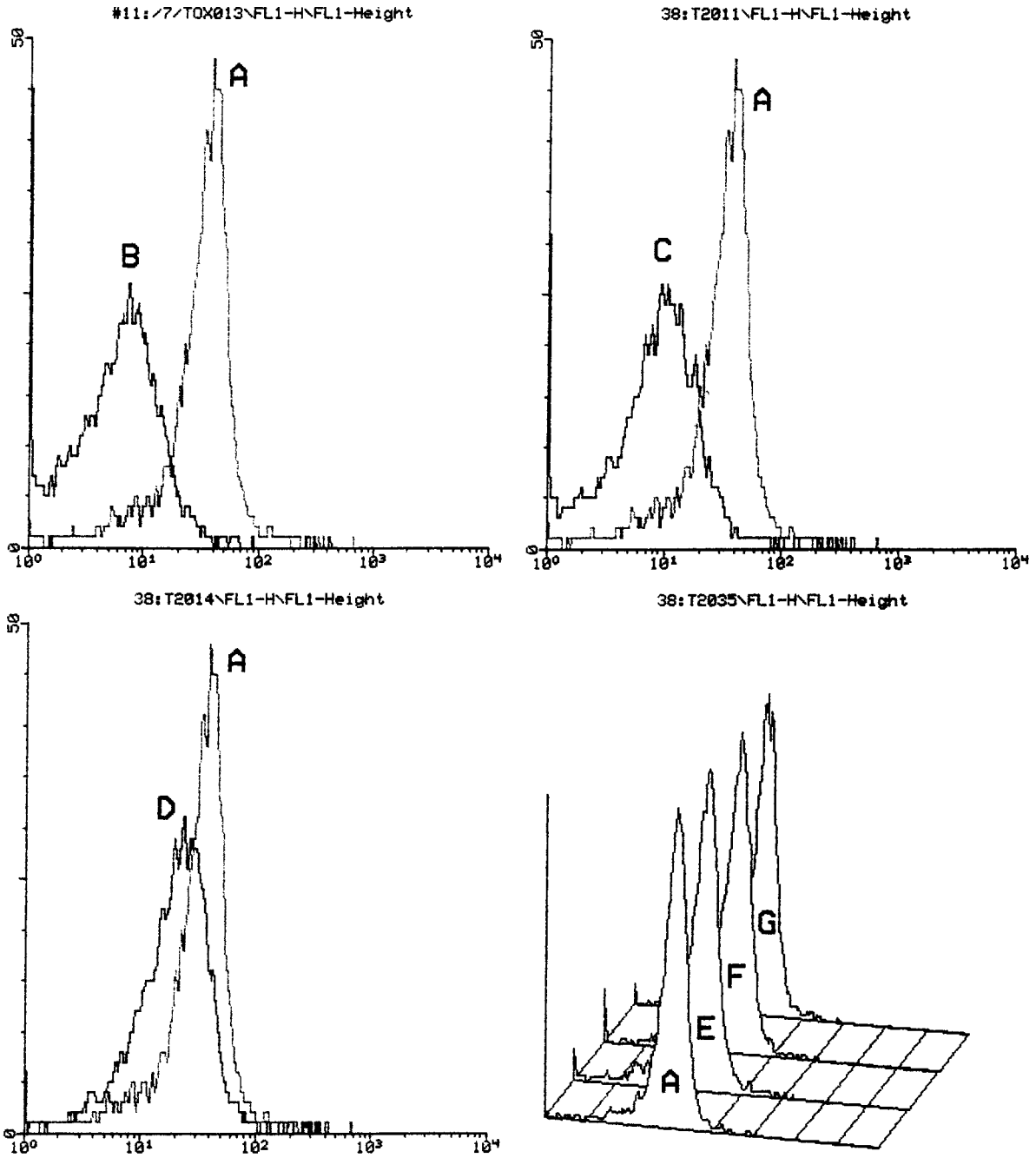

Fig. 2. Cytofluorimetric profile of SE-BSA in the absence of inhibitor (A) and in the presence of 10 μg/ml of SE-BSA (B), SE (C), ISE (D), and derivatives **7** (E), **6** (F), and **14** (G). Abscissa: relative change in sample fluorescence intensity. Ordinate: number of particles.

Seiridium spp. The SE structural features are the $\Delta^{\alpha,\beta}$-unsaturated γ-lactone and the hydroxylated heptyl side chain. The hydroxy group of the latter was converted into the corresponding ketone by Corey's reagent oxidation of SE to 2′-oxoseiridin (**13**). The carbonyl group at C-2′ allowed the binding of **13** to BSA. The resulting Schiff's base of the conjugate was stabilized by $NaBH_4$ reduction.

The conjugate was used to obtain an antiserum recognizing SE. The antiserum was absorbed with BSA and then tested by ELISA and flow cytometry for its capacity to recognize SE (**1**), its natural structural isomer ISE (**2**), 7 derivatives of SE and 6 derivatives of ISE. In the competitive indirect ELISA, the binding activity of SE, ISE and their derivatives was not detectable at any of the concentrations tested. Only the homologous antigen SE-BSA reacted with a value of IC_{50} (i.e., the amount per millilitre of compound required to inhibit the reaction between SE-BSA and antibodies by 50%) higher than 500 μg/ml. In a previous work this

TABLE II. Specificity of the Inhibition Cytofluorimetric Test

Compound	Relative change in sample fluorescence intensity[a]				
	0[b]	0.5[b]	5.0[b]	10[b]	50[b]
SE-BSA	320 ± 2.2	210 ± 1.4	80 ± 0.7	71 ± 0.8	75 ± 1.0
1	317 ± 1.4	270 ± 2.5	230 ± 1.6	196 ± 1.1	190 ± 1.4
13	317 ± 1.4	307 ± 2.8	262 ± 2.4	244 ± 3.1	238 ± 3.0
11	320 ± 3.1	298 ± 2.8	265 ± 2.6	249 ± 3.2	255 ± 2.4
3	320 ± 3.1	305 ± 3.1	284 ± 2.4	258 ± 2.1	260 ± 2.4
2	317 ± 1.4	313 ± 3.2	298 ± 2.8	265 ± 2.1	262 ± 1.8
5	330 ± 2.4	326 ± 3.6	307 ± 3.1	287 ± 2.1	285 ± 2.5
15	317 ± 1.4	318 ± 2.8	310 ± 2.2	289 ± 1.4	283 ± 3.2
4	320 ± 3.1	323 ± 3.1	325 ± 3.4	313 ± 3.2	318 ± 2.4
12	330 ± 2.4	326 ± 3.1	322 ± 2.8	313 ± 3.2	320 ± 4.2
7	320 ± 3.1	322 ± 3.4	322 ± 3.1	317 ± 3.1	318 ± 2.8
10	320 ± 3.1	321 ± 3.2	320 ± 3.8	317 ± 2.7	312 ± 2.8
14	320 ± 3.1	314 ± 3.6	315 ± 3.2	322 ± 4.3	322 ± 3.8
9	320 ± 3.1	327 ± 2.8	318 ± 1.8	323 ± 1.4	325 ± 3.1
6	320 ± 3.1	312 ± 2.6	317 ± 2.2	323 ± 1.4	322 ± 2.6
8	330 ± 2.4	321 ± 3.4	325 ± 3.4	333 ± 4.3	318 ± 3.6

[a]Values (mean channel of fluorescence of samples subtracted by the mean channel of the control) are the average of three experiments ± the standard deviation.
[b]Concentration (μg/ml) of compound used in the test.

method was successful for the identification of cyclopaldic acid, a main toxin produced in vitro by *S. cupressi* [Del Sorbo et al., 1994].

Since ELISA did not allow identification of seiridin we tried the cytometric method. Preliminary experiments showed that the antiserum absorbed with 100 μg of BSA reacted with SE-BSA, but not with BSA, and that the highest fluorescence occurred when the coating of particles was carried out using a 50 μg/ml SE-BSA solution (data not shown). Moreover, in order to optimize the conditions for the competitive indirect cytofluorimetric test, antibodies at different dilutions were incubated with SE-BSA, SE and ISE as inhibitors at concentrations of 0, 10 and 50 μg/ml (Table I).

The results showed that SE and ISE could be differentiated and the difference was particularly evident when the antiserum was diluted to 5×10^{-3}. Increase of the inhibitor concentration from 10 to 50 μg/ml did not cause significant changes in the levels of inhibition. Figure 2 shows the cytofluorimetric profiles obtained using SE-BSA, SE, ISE, and some derivatives (**7, 5,** and **14**) as inhibitors in the competitive assay. The mean channel of sample fluorescence intensity of all compounds tested in this study are reported in Table II.

Both the intra- and inter assay coefficients of variation of the cytometric test were below 5% at all concentrations used. The highest inhibition values in the test were observed when the concentration of the inhibitor reached 10 μg/ml. Higher concentrations of inhibitors (50 μg/ml) did not cause significant reduction of the binding activity. This result probably depends on the avidity of the antibodies which is not sufficient for a complete inhibition of the activity.

As expected, the highest binding activity with antibodies was displayed by the SE-BSA and SE. The activity of seiridin derivatives seems to correlate with the modifications of the two structural features characterizing SE and ISE, that is the integrity of the $\Delta^{\alpha,\beta}$-unsaturated γ-lactone ring and the hydroxy group and its location in the heptyl side-chain. It is interesting to observe that the 2′-acetyl and the 2′-oxoseiridin (**3** and **13**), two derivatives having modified the hydroxy side chain group, as well as *iso*-seiridin, that may be considered a derivative of **1** in which the hydroxy group is shifted from C-2′ to C-3′, retain some activity. This suggests that a reversible modification of the hydroxy group at C-2′ (acetylation or oxidation) as well as its shift at C-3′ (as in **2**) determine only a limited effect on the activity. The decrease of activity was more marked in the assay of the 2′-dansylhydrazoneSE (**15**), probably for a steric hindrance of the alkyl side chain due to the bulky dansyl group attached to C-2′ in this derivative. The furane derivative (**11**), although differing from **1** for its aromaticity, might retain activity because it contains a five membered planar ring with an electronic density similar to that of SE. The 3,4-dihydro-derivativeSE (**5**) showed a weak reactivity, probably due to the saturation of the 3,4-double bond which caused a decrease of the electron density and the loss of planarity of the γ-lactone ring. Derivatives showing a destroyed γ-lactone ring (**7**), as produced by $LiAlH_4$ reductive opening of the ring and its triacetyl derivative (**8**), were inactive. Since the shift of the hydroxy group from C-2′ to C-3′ determines a decrease of the binding activity, the total loss of reactivity of ISE derivatives which also have another structural modification does not surprise. All ISE derivatives having the hydroxy group modified at C-3′ (acetylated or oxidized

as in **4** or in **14**) and those having the hydroxy group unchanged but with some modifications on the γ-lactone ring (hydrogenation or aromatization as in **6** or **12**) were inactive. Finally, as expected, the two derivatives of *iso*-seiridin having an opened γ-lactone ring (**9** and **10**) exhibited no reactivity.

The negative results obtained by ELISA may be correlated to the low avidity of the antibodies as was also observed in the cytometric test. A higher sensitivity of the cytometric method compared to ELISA has been recently found for the detection of the cucumber mosaic virus [Iannelli et al., 1996]. This and our results indicate that flow cytometry may represent an alternative with novel potentialities in comparison to ELISA and other methods.

The presence and the position of the hydroxy group in the heptyl side chain and the integrity of the γ-lactone ring are necessary not only for the biological activity of SE and ISE but also for the recognition of the antibodies raised against the seiridin conjugate. The antibodies are highly specific because little changes in the structure of SE caused a decrease of the reactivity, while compounds with substantial modifications were unreactive. Therefore, the antibodies might be used for the detection of seiridin in complex biological samples. Considering that the type and appearance of the symptoms caused by *Seiridium* species in their host suggest that toxins are produced in the infected tissue plants and are diffused or translocated to the adjacent and even distal parts of the tree [Abbatantuono and Sparapano, 1990; Sparapano et al., 1993a,b], the cytometric assay might be a valuable tool to verify such a hypothesis.

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